

Expression analyses of the mitochondrial complex I 75-kDa subunit in early onset schizophrenia and autism spectrum disorder: increased levels as a potential biomarker for early onset schizophrenia

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Expression analyses of the mitochondrial complex I 75-kDa subunit in early onset schizophrenia and autism spectrum disorder: increased levels as a potential biomarker for early onset schizophrenia

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Abstract Searching for a peripheral biological marker for schizophrenia, we previously reported on elevated mitochondrial complex I 75-kDa subunit mRNA-blood concentrations in early onset schizophrenia (EOS). The aim of this study was to further evaluate the utility of this gene as a potential marker for schizophrenia. Both—schizophrenia and autism—are suggested to be neuronal maldevelopmental disorders with reports of mitochondrial dysfunction and increased oxidative stress. Therefore we have investigated the expression levels of mitochondrial complex I 75-kDa subunit mRNA in whole blood of children with autistic spectrum disorder (ASD) and a group of adolescent acute first-episode EOS patients in comparison to matched controls. We have found that compared to the respective controls only the group of EOS patients—and not the ASD group—showed a significantly altered expression of the

complex I 75-kDa subunit mRNA. Although further studies are necessary to test for the specificity of this marker, our findings point to the potential use of the mitochondrial complex I as a biomarker for schizophrenia.

Keywords Biological marker · Mitochondrial complex I · 75-kDa subunit · Early onset schizophrenia · Autistic spectrum disorder

Introduction

The severe psychiatric condition “schizophrenia” sometimes manifests in children and adolescents as early onset schizophrenia (EOS). The clinical and biological heterogeneity of schizophrenia often hampers a rapid and defin-

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itive diagnosis. Since diagnostic procedures are only based on descriptive behavioral information, a disease marker in peripheral tissue would help to accelerate diagnosis and monitor treatment course. In several studies [1–5] researchers focused their analyses on the complex I of the mitochondrial electron transporter system, due to the repeatedly found abnormalities in cerebral energy metabolism and mitochondrial function in schizophrenia [6–18]. Ben-Shachar et al. described a high sensitivity and specificity for the mitochondrial complex I as a potential biomarker of schizophrenia. They found an increased enzymatic activity of the complex I in schizophrenic patients compared to controls [1]. Furthermore a positive correlation between the enzymatic activity and psychotic symptomatology was found [4].

In a recent study we showed increased levels of complex I mRNA expression for the first time in a rare population of adolescent EOS patients [19]. In schizophrenia research, many studies pointed to a neurodevelopmental etiology of this disorder [20, 21]. There is growing evidence that abnormalities in prenatal or perinatal brain development ultimately result in an increased predisposition to schizophrenia.

Similarly to this disease concept, ASD is supposed to be caused by a neuronal maldevelopment, reflected by cytoarchitectural abnormalities in many brain regions [22–24]. Just like in schizophrenia pathophysiology, a variety of biochemical, anatomical and neuroradiographic studies imply an impairment of brain energy metabolism in ASD [25–30].

The aim of our study was to re-assess the relative utility of mitochondrial complex I 75-kDa subunit mRNA levels as a marker for schizophrenia. In order to assess whether the increased 75-kDa subunit mRNA expression generally reflects an abnormal neuronal development or whether it is more closely related to schizophrenia, we have determined the expression of this gene in a group of ASD children, and in parallel in a group of acute first-episode EOS patients. In both groups mRNA levels were assessed in whole blood cells by real-time PCR in comparison to a respective age- and gender-matched healthy control group. As a positive control we have included the previously examined sample group [19].

Methods

Subjects

Patients were recruited at the hospital for child and adolescence psychiatry and psychotherapy of the University of Wuerzburg and Ulm. The local ethics committees approved this study and all subjects and parents gave their written informed consent.

Typically developing healthy participants were recruited from primary, secondary or grammar school. They were screened for behavioral problems by the Achenbach Child Behavior Check List (CBCL) [31]. Individuals were excluded from the control group if they suffered from a somatic or neurological disease, were taking medication or exhibited abnormal CBCL scores.

We included into this study 10 patients with acute first-episode, neuroleptic-naïve EOS and 10 age- and gender-matched controls of our previous report. These samples were re-analyzed in parallel to the other two groups as a positive control. For demographic details please see [19].

Our second, independent schizophrenia sample consisted of 12 patients (8 males, mean age 16.3 years, age range from 13 years, 6 months to 17 years, 11 months; 4 females, mean age 16.6 years, age range from 15 years, 3 months to 17 years, 6 months) with acute first-episode EOS (see Table 1). Schizophrenia diagnoses (see Table 1) were determined by an experienced child psychiatrist according to ICD-10/DSM-IV criteria. Blood was collected in the acute, if possible neuroleptic-naïve state. Four of the 12 schizophrenic patients were neuroleptic-naïve, 8 had a short period of drug treatment before the first blood withdrawal (see Table 1). The brief psychiatric rating scale (BPRS) [32] was performed concomitantly with the blood withdrawal. This group was age- and gender-matched with a group of 12 control children from our pool of controls (for demographic details on age and CBCL scores see Table 1).

The ASD group consisted of 16 male patients (mean age 13.6 years; age range from 8 years, 2 months to 18 years, 0 months). ASD diagnoses were based on the ICD-10/DSM-IV criteria and determined by an experienced child psychiatrist (see Table 2). Diagnoses were further confirmed by the following measures: The Questionnaire on Behavioral and Social Communication (VSK) [33, 34], the Autism Diagnostic Interview-Research [35, 36], and the Autism Diagnostic Observation Schedule [37–39]. For the ASD questionnaire mean scores and cut-offs see Table 2. ASD patients with a known severe somatic or neurological disorder as well as schizophrenia were excluded from the study. For comorbid diagnoses and medication see Table 2. The ASD group was age- and gender-matched with a group of 16 healthy children (for demographic details on age and CBCL scores see Table 2).

Isolation of total RNA

PAXgene blood RNA kit (PreAnalytiX, Hombrechtikon, Switzerland) was used according to the manufacturers instructions to isolate total RNA from blood followed by DNase treatment (RNase-free DNase Set, Quiagen, Oslo,

Table 1 Demographic statistics of the second, independent EOS sample and the matched control subjects

EOS patients	
Age/gender	
Male (<i>n</i>)	8
Age (years), mean \pm SD	16.3 \pm 1.7
Female (<i>n</i>)	4
Age (years), mean \pm SD	16.6 \pm 1.0
Schizophrenia ICD 10-diagnoses (<i>n</i>)	
F20.0 paranoid schizophrenia	9
F20.2 catatonic schizophrenia	1
F25.0 schizoaffective disorder	2
Psychiatric co-medication (patients, <i>n</i>)	
Drug-naïve	4
On medication	8
Neuroleptics (olanzapine, quetiapine, clozapine, chlorprothixen)	6
Tranquillizer (lorazepam)	3
Mood stabilizer (valproic acid)	1
Others	1
Initial BPRS (score \pm SD)	62.2 \pm 16.4
Control subjects	
Age/gender	
Male (<i>n</i>)	8
Age (years), mean \pm SD	16.0 \pm 1.0
Female (<i>n</i>)	4
Age (years), mean \pm SD	16.6 \pm 1.2
CBCL (score \pm SD)	
Male CBCL total	18.4 \pm 11.5
Internalizing	5.4 \pm 4.3
Externalizing	7.4 \pm 5.3
Female CBCL total	7.7 \pm 4.7
Internalizing	4.7 \pm 4.6
Externalizing	1.3 \pm 1.2

BPRS Brief Psychiatric Rating Scale, *CBCL* Child Behavior Check List

Norway). We evaluated the RNA purity and yield by UV-spectroscopy.

Reverse transcription

For the reverse transcription we used the RETROscript kit (Ambion, Cambridgeshire, UK) with the moloney murine leukemia virus following the manufacturer's instructions using oligo(dT) primers.

Real-time quantitative PCR

Primers were designed with Beacon Designer Software (Biosoft International, Stratagene, La Jolla, CA, USA) and synthesized commercially by Invitrogen (Custom Primers,

Karlsruhe, Germany). Real-time PCR was carried out at the myIQ (Bio-Rad, Hercules, CA, USA) with AbsoluteTM SYBR Green Fluorescein Mix (Abgene, Epsom, UK); each sample in triplicate. Patient samples and matched controls were run on the same plate in one experiment. We used beta-actin as a housekeeping gene (in accordance with our first protocol, [19], for a better comparability). Primer sequences, annealing temperature and product size of the amplified product are shown in Table 3. PCR conditions were the following: initial enzyme activation cycle 95°C (15 min), denaturation 94°C (20 s), annealing TA (30 s), elongation 72°C (30 s), extra step at 80°C to exclude primer dimers (15 s). 45 cycles of denaturation, annealing, elongation and the extra step were used. For the optimization of the assay conditions and specificity we performed melt curve analysis and gel electrophoresis. We also analyzed controls without reverse transcriptase and no template controls. Our standard curves were performed in triplicates and primer efficiency was determined to be within a suitable range for the use of the 2^{ΔΔCT} method. Real time quantitative-PCR products were assessed in a 2% agarose gel containing SYBR Save DNA gel stain (Invitrogen) with a 100 bp DNA ladder (Promega).

Data analysis and statistics

To compare gene expression levels we applied the 2^{ΔΔCT} method using the Biorad iQ5 Optical System Software version 2.0. When comparing age- and gender-matched patient and control groups, two-tailed *t*-tests were used, after normal distribution was confirmed by Kolmogorov–Smirnov test. A two-tailed type I error rate of 5% was chosen.

For the analysis of the sensitivity and specificity of our potential marker we calculated the 2^{ΔΔCT} difference of each matched patient-control-pair and determined the cut off that separated best the EOS from the ASD group (by receiver operating characteristic-curves in SPSS).

Results

All patients of our two independent schizophrenia groups had a severe disease. Patients of our previously tested sample [19], which we have included as positive control, had a mean initial BPRS value (\pm standard deviation; SD) of 58.1 \pm 10.6. In our new, independent schizophrenia group the mean initial BPRS value was 62.2 \pm 16.4 (see Table 1). The two groups did not differ in their mean age \pm SD, with 16.5 \pm 3.3 years in the first and 16.4 \pm 1.4 in the new sample. There were slightly more female patients in the second group (20% in the first versus 33.3% in the new sample). The main diagnosis in both

Table 2 Demographic statistics of the 16 male ASD patients and the matched control subjects

ASD patients (<i>n</i> = 16, male)	
Age (years), mean \pm SD	13.6 \pm 3.5
ASD ICD 10-diagnoses (<i>n</i>)	
F84.0 Childhood autism	2
F84.1 Atypical autism	5
F84.5 Asperger's syndrome	9
Comorbid ICD 10-diagnoses (<i>n</i>)	
F31.3 Bipolar affective disorder, current episode mild or moderate depression	1
F42.2 Mixed obsessional thoughts and acts	1
F80.0/ F81.1 Specific speech articulation disorder/expressive language disorder	1/1
F83 Mixed specific developmental disorder	1
F90.0/90.1 Disturbance of activity and attention/hyperkinetic conduct disorder	8/1
F93.3 Sibling rivalry disorder	1
F94.0 Elective mutism	1
F95.1/ F95.2 Chronic motor or vocal tic disorder/combined vocal and multiple motor tic disorder	1/1
F98.0/ F98.1 Non-organic enuresis/encopresis	1/1
Psychiatric co-medication (patients, <i>n</i>)	
Methylphenidate (including Medikinet ret. [®] , Concerta [®])	6
Amphetamine	2
Atomoxetine	1
Fluoxetine	2
Sertraline	1
Lithium acetate	1
Risperidone	1
Tiapridex	1
Autistic features (score \pm SD)	
FSK	19.9 \pm 6.1
ADOS sum of communication and social interaction (autism cut-off = 10, autistic spectrum disorder = 7)	11.5 \pm 4.4
ADI-R social interaction (cut-off 10)	19.9 \pm 8.4
ADI-R communication and language (cut-off for speaking children = 8)	15.0 \pm 4.9
ADI-R repetitive behaviours and stereotyped pattern (cut-off = 3)	5.8 \pm 3.1
ADI-R abnormal development before the age of 3 years (cut-off = 1)	2.5 \pm 1.7
Control subjects (<i>n</i> = 16, male)	
Age (years), mean \pm SD	14.2 \pm 2.1
CBCL (score \pm SD) CBCL total	13.8 \pm 10.0
Internalizing	3.4 \pm 3.5
Externalizing	5.6 \pm 4.4

ADI-R Autism Diagnostic Interview-Research, ADOS Autism Diagnostic Observation Schedule, ASD autistic spectrum disorder, CBCL Child Behavior Check List, FSK Fragebogen zur Sozialen Kommunikation [original version: Social Communication Questionnaire (SCQ)]

Table 3 Primer sequences, annealing temperatures and PCR product sizes

Name	Gene bank code (NCBI, Gene)	Primer sequences 5' to 3'	Annealing temperature	Product size
75-kDa-subunit = NDUFS1	<u>BC030833</u>	f-ATGTGCCTTGTTGAAATTGAGAAAG r-GCATAGGGCTTAGAGGTTAGGG	62°C	482
Beta-actin	<u>BC002409</u>	f-TGAAGTGTGACGTGGACATCCG r-GCTGTCACCTTCACCGTTCCAG	62°C	444

NDUFS1 NADH dehydrogenase (ubiquinone) Fe-S protein 1

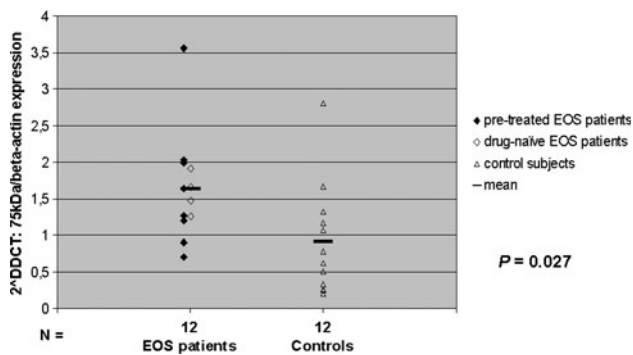


Fig. 1 Mitochondrial complex I 75-kDa subunit gene expression in the second, independent early onset schizophrenia patients sample (pre-treated and drug-naïve) and the matched control subjects

samples was the ICD-10 F20.0, paranoid schizophrenia (80 versus 75%). One difference of the two groups was that in our new, independent sample 4 of the 12 patients were drug-naïve and 8 were on a short period of neuroleptic medication at the time of blood withdrawal.

The third group we have investigated included ASD patients. In the ASD group, the requested cut-offs of the state of the art standardized autism manuals were clearly exceeded (all data see Table 2).

In our pool of controls we found a correlation between age and $2^{\Delta\Delta\text{CT}}$ values with a higher 75-kDa/beta-actin ratio in the younger persons ($n = 24$; $r = -0.491$, $P = 0.015$). Therefore our respective control groups were carefully age-matched. Comparing the twelve EOS adolescents of our new, independent schizophrenia sample with age- and gender-matched controls, revealed a significantly increased 75-kDa subunit mRNA expression (ratio to beta-actin; $2^{\Delta\Delta\text{CT}}$ values: schizophrenia 1.6 ± 0.7 versus controls 0.9 ± 0.8 ; $P = 0.027$; for the $2^{\Delta\Delta\text{CT}}$ values and means of pre-treated and drug-naïve patients see Fig. 1) similarly to the newly investigated positive control group, where we could reproduce the increased 75-kDa subunit mRNA expression in a different laboratory, by a different investigator (ratio to beta-actin; $2^{\Delta\Delta\text{CT}}$ values: schizophrenia 2.3 ± 2.1 versus controls 0.8 ± 0.4 ; $P = 0.053$). The $2^{\Delta\Delta\text{CT}}$ values and mean of the two combined EOS samples and two control samples are shown in Fig. 2 (ratio to beta-actin; $2^{\Delta\Delta\text{CT}}$ values: schizophrenia 1.9 ± 1.2 versus controls 0.9 ± 0.8 ; $P = 0.003$). Comparing drug-naïve with pre-treated EOS patients in the second independent EOS sample [drug-naïve ($n = 4$) versus pre-treated EOS ($n = 8$); $P = 0.816$; for a graphical presentation of the respective $2^{\Delta\Delta\text{CT}}$ values see Fig. 1] as well as in the combined EOS sample [drug-naïve ($n = 14$) versus pre-treated EOS ($n = 8$); $P = 0.583$] we could not detect any 75-kDa gene expression differences.

Comparing the ASD group and age- and gender-matched controls, we could not detect any difference in mRNA

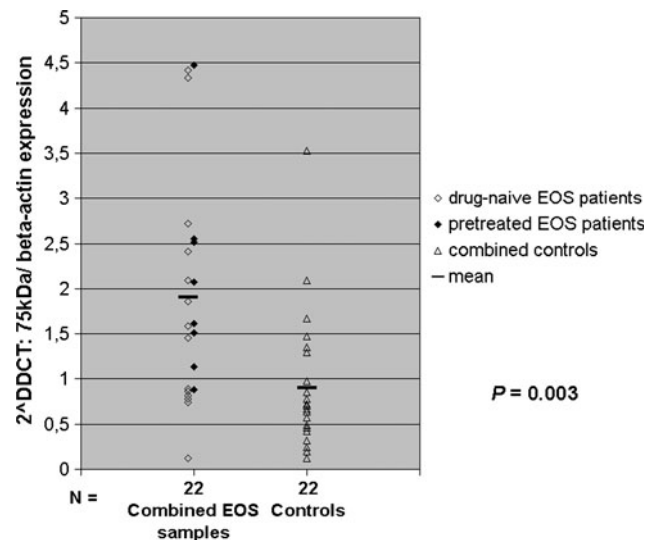


Fig. 2 Mitochondrial complex I 75-kDa subunit gene expression in the two combined EOS patients samples (pre-treated and drug-naïve) and the combined control groups

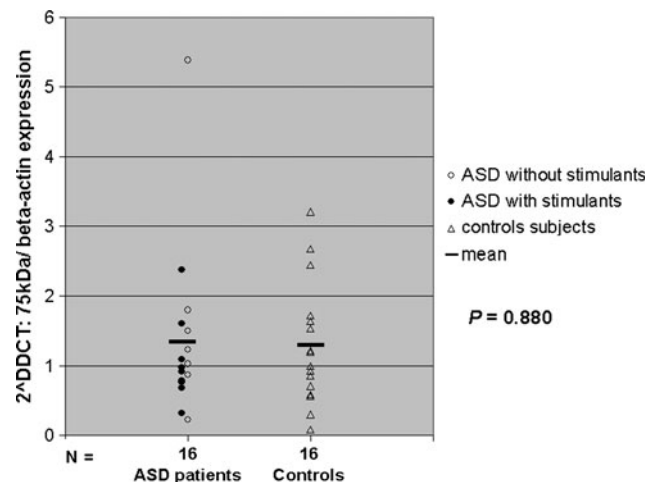


Fig. 3 Mitochondrial complex I 75-kDa subunit gene expression in the ASD patients sample (patients with and without stimulants) and the matched control subjects

levels of the complex I 75-kDa subunit (ratio to beta-actin; $2^{\Delta\Delta\text{CT}}$ values: ASD 1.3 ± 0.9 versus controls 1.3 ± 1.2 ; $P = 0.880$; see Fig. 3), neither when comparing the ASD patients on stimulant medication with the untreated ones ($P = 0.460$; for a graphical presentation of the respective $2^{\Delta\Delta\text{CT}}$ values see Fig. 3).

We found a sensitivity of the potential biomarker of 0.82 and a specificity of 0.63.

Discussion

The aim of our study was to further evaluate the utility of mitochondrial complex I 75-kDa subunit mRNA levels as a

potential biomarker for schizophrenia. In order to assess whether the increased 75-kDa expression generally reflects an abnormal neuronal development or whether it is more closely associated with schizophrenia we have determined the expression of this gene in a group of ASD children, and in parallel in a group of acute first-episode EOS patients. In both groups mRNA levels were assessed in whole blood cells by real-time PCR in comparison to a respective age- and gender-matched healthy control group. As a positive control we have included the previously examined sample group [19]. As all EOS and ASD patients showed a strong symptomatology—assessed by the state of the art manuals—suggest the comparison of the 75-kDa gene expression in these two neuronal maldevelopmental disorders for evaluating the utility of this gene as a potential marker for schizophrenia.

In our study we observed an increased 75-kDa subunit expression in our two independent samples of EOS. In the ASD group we were not able to detect any altered gene expression. Our potential marker separated our EOS and ASD groups with a sensitivity of 0.82 and specificity of 0.63. For a final evaluation of specificity further studies on patients with other psychiatric disorders are certainly needed. To increase sensitivity and specificity, probably a set of different biomarkers (such as different gene variants, markers on the level of mRNA and protein expression in combination with functional imaging data etc.) should be used. Such specific biomarkers would allow an early diagnosis and treatment monitoring of schizophrenia.

There are several reports, that peripheral gene expression/activity is correlated with gene expression/metabolic function in the central nervous system (CNS). Sullivan et al. [40] performed a study on the comparability of gene expression in blood and brain. It was found that about half of a set of candidate genes relevant to schizophrenia were expressed in both whole blood and prefrontal cortex tissue. Furthermore it was found, that the complex I activity in platelet mitochondria was positively correlated with the cerebral glucose metabolism in basal ganglia and thalamus, two brain regions implicated in schizophrenia [2]. Findings such as these affirm that it is reasonable and useful to search for disease markers in peripheral tissue as surrogate for central processes, as in our study.

Although this study might be limited by the small sample size, our results are in line with former reports of an increased gene expression and activity of mitochondrial complex I in schizophrenia. Mitochondrial complex I locates at the mitochondrial inner membrane and is composed of 45 different subunits. Our candidate gene, the 75-kDa subunit, is the largest iron–sulfur non-catalytic transmembrane protein of the complex I [1, 4]. It has NADH dehydrogenase and oxidoreductase activity and transfers electrons from NADH to the respiratory chain. Functional

abnormalities in this subunit might impact on the electron transport of NADH to oxygen and finally on the cellular ATP production, leading to various brain dysfunctions, such as seen in schizophrenia pathophysiology. Ben-Shachar et al. described a high sensitivity and specificity for potential mitochondrial complex I markers in adults with schizophrenia [1, 4]. They found an increased enzymatic activity of the complex I in schizophrenic patients compared to controls and patients with affective disorders [1]. Dror et al. [4] observed concordantly, that increased mitochondrial complex I activity was associated with psychotic symptomatology, while its decrease was observed in patients with residual schizophrenia. There are also several reports of brain region-dependent altered gene expression of mitochondrial complex I subunits in post-mortem tissue of schizophrenic patients. Association studies additionally pointed to a modulating effect of complex I gene variants in schizophrenic psychosis [41].

As a possible confounding factor of our results, gene expression of the 75-kDa subunit might be influenced by medication. Most of our ASD patients were on medication, mainly on stimulant drugs. There is little data published on methylphenidate effects on the respiratory chain. In one report chronic methylphenidate administration increased the CNS expression of mitochondrial complex II and IV genes in a tissue dependent way [42]. Under chronic amphetamine abuse, an increased oxidative stress was repeatedly found (e.g., [43]), suggesting altered mitochondrial function. Half of our second, independent EOS sample were under a short duration antipsychotic medication at time of blood withdrawal (see Table 1). Under chronic antipsychotic treatment, Casademont et al. [44] monitored a reduction of mitochondrial complex I activity in peripheral blood mononuclear cells in comparison to the drug-naïve status. There was also a decrease of complex I activity seen when antipsychotics were added, *in vitro*, to mitochondria isolated from human and rat brains [1, 45, 46]. However, the observation of tissue-specific effects of neuroleptics on complex I activity *in vivo* (activity reduction in rat brain, but not in muscle) might explain the varying results [47, 48]. Several reports disproved a possible confounding influence of antipsychotics on complex I expression. Whatley et al. [49, 50] reported that neuroleptics did not modify the complex I NADH-coenzyme Q reductase nor rotenone-resistant NADH cytochrome C reductase activity in postmortem frontal cortex of schizophrenic patients. Ben-Shachar et al. [1] found a similar complex I activity in unmedicated and medicated patients. Also our previous results confirmed these observations, as the increased 75-kDa expression was seen in the neuroleptic-naïve patients as well as after a three month antipsychotic treatment [19]. In our current study we observed the increased 75-kDa subunit gene expression in our new,

independent EOS sample in the drug-naïve state as well as under a short antipsychotic treatment. Observing elevated 75-kDa subunit mRNA levels in the medicated and unmedicated acutely and chronically psychotic state, but not in residual schizophrenia [1, 4, 19] suggests the mitochondrial 75-kDa-subunit as a potential state marker of schizophrenia.

Conclusion

In conclusion, our results strengthen the role of the mitochondrial complex I as a potential peripheral biomarker for schizophrenia. The observed increase in 75-kDa subunit mRNA expression is in line with other reports on altered complex I activity in schizophrenic psychosis and further findings of increased oxidative stress in schizophrenia pathophysiology [18, 51]. Although further studies are needed to confirm the specificity it might become a convenient supporting peripheral marker for an early clinical diagnosis and treatment of this disease.

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